Cold tolerance during larval development: effects on the thermal distribution limits of *Leptinotarsa decemlineata*

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Abstract

Insects' cold tolerance during their development is a surprisingly understudied subject in ecology, despite the fact that subzero temperatures during the growing season are common at high altitudes and latitudes. Subzero temperatures can have detrimental effects on organisms, restricting a species' range. This study addresses the question whether night frosts during the growing season have an instant or delayed negative impact on larval mortality of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). We also tested whether populations from the centre (Poland) and margins (Russia) of the distribution range of *L. decemlineata* differ in their responses to subzero exposure and a low rearing temperature. Larvae of three ages were subjected to a subzero temperature (-4 °C for 3 h simulating night frost) twice, after which they were reared on a fluctuating temperature regime of 10–15 °C. These rearing conditions imitated cool summer temperatures beyond the beetles' current range, such as in Finland. Individuals of both populations were highly cold tolerant, as only 3.1% of larvae died immediately following the subzero treatment. Nonetheless, the low rearing temperature was harmful to beetles of both populations. It caused high larval (ca. 90%) and overwintering (ca. 80%) mortality. As beetle performance was affected solely by rearing temperature, low temperatures during the growing season rather than night frosts apparently retard the beetle's northern expansion.

Introduction

A precise understanding of the factors that limit a species range is necessary to be able to predict changes in species distribution (Griffith & Watson, 2006). Temperature plays a major role in defining the geographic distribution of insect species (Gaston, 2003; Chown & Nicolson, 2004). The thermal range of a species is defined by the base temperature needed to complete their development and the lowest temperatures at which adults can survive. However, climatic conditions in different ranges do not simply consist of higher or lower average temperatures. Insects may be exposed to temperature extremes even in the middle of the growing season (i.e., subzero temperatures due to night frost) especially at high latitudes and altitudes. As sudden frosts can be common, they can determine insect distribution and limit growth and reproduction (Jenkins & Hoffmann, 1999; Inouye, 2000; Tesar & Scriber, 2002). Although the establishment of insects in a given area might be restricted by thermal extremes more than by average temperature, the importance of extreme environmental conditions in defining species borders has been neglected in many studies (Gaston, 2009).

Testing the thermal limits of species borders involves examining all of these factors. Although many physiological studies have examined insect cold tolerance during overwintering (e.g., Tullett et al., 2004; Turnock & Fields, 2005), perhaps because of its reputation as a key factor limiting species distribution (e.g., Gaston, 2003; Crozier, 2004; Tullett et al., 2004; Valosaari et al., 2008), relatively little is known about insect cold tolerance during

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development. Studies on cold tolerance during the growing season are needed because the responses of diapausing individuals to cold cannot be compared with those of non-diapausing individuals. Diapausing organisms are typically more tolerant than non-diapausing ones (e.g., Sinclair, 1997; Worland & Convey, 2001). Moreover, cold tolerance changes both with season (van der Merwe et al., 1997; Worland & Convey, 2001; Koch et al., 2004; reviewed in Danks, 2005) and developmental stage (Klok & Chown, 2001). Thus, further information about cold tolerance during the growing stages of an insect is needed to render more reliable predictions about their ability to invade new areas with harsh temperature conditions (Gaston, 2009). Here, we examine the factors involved in the thermal distribution limits of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), by considering conditions beyond their current limits during their developmental stages.

Leptinotarsa decemlineata has spread across large areas worldwide including Europe, where its northern distribution limit in Russia is around 61°N (EPPO, 2006). However, there are still areas, like Scandinavia, where the beetle's host plant, potato, is cultivated but which do not have permanent beetle populations. These unoccupied areas have average summer temperatures that would enable beetles to complete their development, indicating ecologically suitable invasion areas (Boman et al., 2008; Lyytinen et al., 2008). Low summer temperatures (12–15 °C) (Drebs et al., 2002) occurring every few years, however, may not allow the beetles to successfully complete their development in these areas (optimum temperature for *L. decemlineata* development is 28 °C; Ferro et al., 1985). Moreover, the impact of night frost during the growing season, which is common in the species' theoretical invasion range, on the beetle's performance is unknown.

We tested the direct and delayed effects of subzero temperatures on survival and life-history traits of various developmental stages of non-diapausing *L. decemlineata* from the centre (Poland) and margins (Russia) of the beetles' distribution range. Beetles were exposed to night frost and reared at the low temperatures that occur beyond their current northern range in Europe. We used very large sample sizes (total n = 881), as larval survival was expected to be very low near the threshold temperature (Ferro et al., 1985; Tauber et al., 1988). The large sample size helped us to assess whether even a fraction of individuals can survive to adulthood under suboptimal conditions; we can thereby gain more reliable data on the ability of the species to colonize new areas with harsh temperature conditions.

In this study, we addressed the following questions: (1) Do night frosts have an instant or delayed negative impact on the larval mortality of *L. decemlineata*? Early developmental stages of insects are expected to be the most susceptible to subzero temperatures (see Neven, 2004). Dormant adult beetles are relatively cold-tolerant (Boiteau & Coleman, 1996), but the cold tolerance of other developmental stages is not known. (2) Are beetles able to complete their life cycle at low temperatures (mean 10–15 °C)? As we reared beetles under conditions found in areas beyond their current northern border, their potential to expand their range northward in Europe can be estimated on the basis of these results. (3) Do populations from the centre (Poland) and margins (Russia) of the distribution range of *L. decemlineata* differ in their responses to subzero exposure and low rearing temperatures? Individuals from higher latitudes are usually more cold tolerant than those from lower latitudes (see Chen & Kang, 2004; Bahrndorff et al., 2006). The differences between populations would suggest directional selection for increased cold tolerance (Hoffmann & Blows, 1994), indicating that subzero temperatures may be important as a limiting factor for a species' range.

Materials and methods

Beetles

Russian beetles represent the northernmost (marginal), and Polish the central populations of *L. decemlineata* in Europe (EPPO, 2006). The adult beetles used were overwintered descendants of field-collected individuals from Russia (from four sites in an area of 150–200 km) around latitude 59°N (northern Russia) and Poland (one site) around latitude 54°N. Field-collected beetles were brought to the laboratory and their offspring were maintained at a constant 23 °C and, after overwintering at 5 °C, they were paired randomly within populations (Russia: 39 females, 24 males; Poland: 17 females, 17 males). Eggs from these matings were used in the experiment. Experimental larvae used were the second laboratory generation, thereby minimizing possible maternal effects (Mousseau & Dingle, 1991).

Average June–August temperatures at the collection sites in Russia are in the range of 15–18 °C (observation site: St. Petersburg) and in Poland, 16–18 °C (nearest observation site: Szczecin), based on climatological data from http://www.eurometeo.com. Not only lower temperatures, but also night frosts during the growing season are more likely in Russia than in Poland. The Russian population has therefore potentially experienced selection for increased cold tolerance.

Rearing conditions

Egg clutches and larvae were kept individually in Petri dishes lined with paper and potato leaves in a controlled

environmental chamber (Type B1300; Weiss Umwelttechnic, Reiskirchen-Lindenstruth, Germany; temperature tolerance range: 0.5 °C). Egg clutches were kept separately at a constant temperature of 23 °C until they hatched. Larvae were maintained under a variable regime in which the temperature was 10 °C during the 4 h of dark, warmed up to 15 °C during the 2 h of dawn, kept at 15 °C throughout the 16 h of light, and returned to 10 °C during the 2 h of dusk (average 13 °C). This photoperiod corresponds to that of the northern range of *L. decemlineata* (based on data from http://www.gaisma.com). The temperature regime reflects cold summer conditions in Finland (Drebs et al., 2002), conditions beyond the beetles' current northern border. The average summer temperature in central Finland (62°N, 25°E) falls below 14 °C approximately every fourth summer (based on 60 years climatological data from the Finnish Meteorological Institute; N Niinimäki, pers. comm.). In 2008, for example, it was just 13.4 °C.

Cold tolerance of eggs

We studied the cold tolerance of the eggs by assessing their hatching success after subzero exposure. In total, 48 egg clutches from 14 females from the marginal population were separated into four groups; one control group (n = 12) and three experimental groups of eggs divided according to age: 2–3 (n = 10), 5–6 (n = 15), or 8–9 (n = 11) days old. This was done to determine whether susceptibility to −4 °C varies with egg age. Eggs in Petri dishes lined with blotting paper were transferred directly from the rearing temperature to −4 °C (mean ± SE = −4.3 ± 0.05 °C) for 6 h on two consecutive days during the night period. This subzero treatment was equivalent to night frosts, which occur in the summertime at northern latitudes. In central Finland (62°N, 25°E) and southeastern Finland (61°N, 28°E), there are on average 13.5 and 6.3 nights with subzero temperatures in the summer, respectively (based on over 40 years climatological data from the Finnish Meteorological Institute; H Pohjanoksa, pers. comm.). After exposure, eggs were maintained under the rearing conditions until hatching or else until they were 14 days old. Control eggs were kept under rearing conditions until they hatched.

Cold tolerance of larvae

To test whether larval cold tolerance differs with developmental stage (see Neven, 2004), we exposed larvae of different ages to −4 °C during the night period. First instars of equal weight were divided into three experimental groups within populations, according to the age at which they were treated (1–2 days old: $n_{marginal}$ = 130, $n_{central}$ = 73; 9–10 days old: $n_{marginal}$ = 150, $n_{central}$ = 73; 19–20 days old: $n_{marginal}$ = 166, $n_{central}$ = 75), and one control group (no cold exposure; $n_{marginal}$ = 142; $n_{central}$ = 72). Siblings (marginal: n = 19 females; central: n = 13 females) were divided equally among these groups, creating a split-brood design. Larvae, in individual Petri dishes, were transferred directly from the rearing conditions to −4 °C for 3 h, after which they were returned to the rearing conditions. The subzero treatment was repeated the following day. Treatment and control larvae were maintained under rearing conditions until adulthood and fed daily with fresh potato leaves (variety Van Gogh, grown in a greenhouse). We measured their immediate larval mortality, development time, weight at emergence (± 1 mg) (on an AM100 balance; Mettler-Toledo Inc., Columbus, OH, USA), and larval-to-adult survival.

Overwintering mortality

To examine the delayed impact of subzero treatment, we recorded the adult overwintering success of both control and experimental individuals. After emergence, adults were allowed to eat for 3 weeks and were then moved to plastic pots (3.8 cm in diameter) containing peat for overwintering. Beetles were fed with potato leaves and maintained at the rearing temperature until they burrowed into the soil, when they were transferred to the overwintering conditions. Beetles overwintered in the dark at a constant 5 °C from September to May. The peat was watered once a month to maintain adequate moisture. Overwintering mortality was calculated by dividing the number of dead beetles at the end of winter by the number of beetles alive at the beginning of winter. Overall survival was also calculated by dividing the number of survivors after overwintering by the number of larvae at the beginning of the experiment. We were thereby able to analyse the combined effects of low summer temperatures and cold shock on overwintering success.

Statistical analysis

Egg hatchability (%) was arcsine √x transformed, and analysed with one-way ANOVA. Larval-to-adult mortality and overwintering mortality data were analysed with a binary logistic regression model with survival (alive/dead) as dependent variable and population and treatment as covariates. The potential presence of differences among experimental groups and between populations for development time and adult weight was analysed with ANCOVA. The hatching weight was taken as covariate, as it affects many life-history traits (see, e.g., Mappes et al., 1996). Population and experimental group were entered as fixed factors. As a result of the significant interaction between population and treatment in development times, comparisons between populations and among

experimental groups were conducted with univariate tests. Least Significant Difference (LSD) post-hoc tests were used to compare the different treatments. All statistics were computed with SPSS 14.0 (SPSS, Chicago, IL, USA).

Results

Effects of subzero treatment

Subzero treatment had no effect on egg hatchability (one-way ANOVA: $F_{3,44} = 0.129$, P = 0.94). Hatchability was 54.8% for the control eggs and 47.3 (2–3 days old), 54.9 (5–6 days), and 55.9% (8–9 days) for the experimental groups. Furthermore, subzero treatment had only minor effects on larval mortality. Only 21 out of 667 larvae (3.1%) died immediately after exposure to subzero treatment. Thus, subzero treatment did not have any significant effect.

Larval-to-adult mortality

Larval-to-adult mortality was very high but similar for all larval stages, irrespective of whether larvae were exposed to −4 °C or not (binary logistic regression: Wald = 0.543, d.f. = 3, P = 0.91). This indicates that larval mortality was not due to subzero treatment. Both populations suffered equal overall larval-to-adult mortality (marginal 90.5%, central 92.2%; Wald = 0.001, d.f. = 1, P = 0.98). In total, 802 out of 881 individuals (91.0%) died before the onset of winter diapause. The interaction between treatment and population was not significant (Wald = 2.635, d.f. = 3, P = 0.45).

Beetle performance

As a result of the high mortality (>90%), the analysis of life-history variables is only suggestive. Sexes did not differ in development time (one-way ANOVA: $F_{1,77} = 0.105$, P = 0.75) but there was a significant interaction between population and subzero treatment for development time (two-way ANCOVA: $F_{3,70} = 3.541$, P = 0.020; Table 1).

This lends support to the hypothesis that the two populations respond differently to subzero treatment. Development times differed among larval treatment groups in the central population (univariate test: $F_{3,70} = 3.533$, P = 0.019), but not in the marginal population ($F_{3,70} = 1.076$, P = 0.37). Larvae of the central population that were exposed to −4 °C at the age of 19–20 days developed more slowly than both control larvae (LSD: P = 0.010) and other experimental individuals (19–20 vs. 1–2 days: P = 0.002; 19–20 vs. 9–10 days: P = 0.018). Other differences were not significant (P≥0.41 for all tests). Hatching weight (covariate) had significant effects only in the marginal population ($F_{1,51} = 6.735$, P = 0.012; central: $F_{1,18} = 0.023$, P = 0.881), where the lightest individuals had the slowest development. Development times between populations also differed (Table 1). Individuals from the marginal population developed significantly faster than those from the central population in the comparison of control ($F_{1,70} = 7.003$, P = 0.010) and 19–20-day-old larvae experimental groups ($F_{1,70} = 22.608$, P<0.001). All other differences were non-significant (P≥0.096 for all tests).

Adult males weighed less than females (ANCOVA: $F_{1,60} = 15.179$, P<0.001) (Table 2). There were no effects of population ($F_{1,60} = 0.001$, P = 0.99), subzero treatment ($F_{3,60} = 1.275$, P = 0.29), or hatching weight (covariate) ($F_{1,60} = 0.011$, P = 0.92) on adult weight. Moreover, all interactions were not significant (P≥0.076 for all tests).

Overwintering mortality

Overwintering mortality, i.e., the proportion of dead beetles from the start of overwintering to its end, was high (79.2%) but similar in both control and treatment groups (binary logistic regression: Wald<0.001, d.f. = 3, P = 1.0). This indicates that subzero treatment did not have a delayed negative impact on beetles that increased their overwintering mortality. Geographical variation in overwintering mortality was not detected, as marginal (77.1%) and central (84.6%) populations suffered equally high

Table 1 Mean development time (days ± SE) for control and cold-exposed *Leptinotarsa decemlineata* larvae from the marginal and central population

Age of larvae when treated (days)	Marginal (Russia)	Central (Poland)
No exposure	54.0 ± 0.81 (12)	59.0 ± 1.18 (6)
1–2	56.33 ± 0.84 (12)	58.25 ± 1.33 (8)
9–10	56.36 ± 1.30 (14)	59.83 ± 1.52 (6)
19–20	54.72 ± 0.92 (18)	66.0 ± 2.52 (3)

Sample sizes are in parentheses.

Table 2 Adult weight (mg ± SE) of control and cold-exposed *Leptinotarsa decemlineata* larvae

Age of larvae when treated (days)	Females	Males
No exposure	111.29 ± 3.32 (7)	89.46 ± 4.14 (10)
1–2	109.86 ± 4.23 (8)	88.82 ± 3.55 (12)
9–10	101.45 ± 2.14 (11)	90.32 ± 4.37 (9)
19–20	95.43 ± 5.54 (6)	92.45 ± 3.08 (14)

Populations have been pooled; sample sizes are in parentheses.

mortality (Wald = 0.357, d.f. = 1, P = 0.55). Overall survival for all individuals used in this experiment was low (i.e., survival from first instar to overwintered adult): only 10 out of 881 (1.1%) survived to the following breeding season. The interaction between treatment and population was not significant (Wald = 0.039, d.f. = 1, P = 1.0).

Discussion

Subzero treatment had little effect on *L. decemlineata*. It had no effect on egg hatchability and the immediate larval mortality due to subzero treatment was very low (3.1%). Furthermore, it was similar in all larval stages, suggesting equal high cold tolerance during development. The result was unexpected, as young larvae have been shown to be the most vulnerable in some species (e.g., Neven, 2004) although different development stages can be equally cold tolerant in others (Koch et al., 2004). These results might be explained by differences either in cold hardiness or in the methods used. Mortality immediately following subzero exposure was expected to be higher, as our treatment was not accompanied by an acclimation period prior to the transfer to −4 °C (Kelty & Lee, 1999; Powell & Bale, 2004; Rako & Hoffmann, 2006). Cold tolerance is sometimes improved by a low overall rearing temperature. Rako & Hoffmann (2006) exposed *Drosophila melanogaster* Meigen to subzero temperatures and found that flies reared at 19 °C had lower mortality than those reared at 25 °C. However, subzero treatment did not cause increased mortality in *L. decemlineata* larvae reared at 23 °C (Heikkilä & Perälä, 2007), indicating that larvae are cold tolerant even when reared at higher temperatures than in the experiment presented here. Our unpublished data further indicates that larvae can withstand even longer exposure to subzero temperatures, as they survived 5 days at −4 °C.

Larval-to-adult mortality was very high (>90%) which could not be explained by exposure to subzero temperature as it was similar in the control individuals. It was most likely due to the low rearing temperature. Under these conditions, larvae were probably not able to eat sufficiently (Ferro et al., 1985; Fei & Henderson, 2002; Yee & Murray, 2004) and consequently, they suffered a period of malnutrition resulting in high mortality and small body size. This conclusion is supported by the fact that these beetles weighed less on average than beetles reared at 23 °C (females: 106 ± 20.1 mg; males: 95 ± 15.4 mg; mean ± SE) (Boman et al., 2008). Small size, in turn, is often associated with high overwintering mortality in insects (Barron & Wilson, 1998; Smith, 2002) because of insufficient body lipid reserves (Pullin, 1987; Zhou et al., 1995). Indeed, overwintering mortality

was higher than that of beetles reared under more favourable conditions (average 23 °C: 40–60%; Boman, 2008). As the probability of establishment after invasion increases with the propagule size (Grevstad, 1999; Memmott et al., 2005), the high larval-to-adult and adult overwintering mortality might limit beetles' ability to sustain populations in northern climates (Valosaari et al., 2008). A few beetles did survive to adulthood, however, which makes it at least theoretically possible for beetles to establish populations under these harsh conditions. There are cases in which the invasion of a single mated female has resulted in successful establishment (Grevstad, 1999; Memmott et al., 2005). Moreover, high mortality could be compensated by high fecundity as one female can produce up to 1 500 eggs during a breeding season (Jansson et al., 1989; S Boman, pers. obs.). On the other hand, low ambient temperature might decrease the fitness of surviving beetles, diminishing the chances of successful establishment as fecundity in insects is associated with body size (Speight et al., 2008). Low fecundity, in turn, might decrease the probability of successful invasion (Kolar & Lodge, 2001; Sakai et al., 2001; Gaston, 2009).

Low average temperatures could result in cold adaptation, which produces individuals with higher fitness than those beetles from warmer climates reared at low temperatures (Karlsson & Wiklund, 2005). Local adaptation to cold tolerance was not detected. The populations studied differed only in development times, as the beetles from the marginal area developed faster than those from the central area (Table 1). Despite the small sample size due to high larval mortality, these results were consistent with results from an earlier study in which we tested the performance of individuals from four European populations and in which we showed that the beetles of northern origin developed faster than those from the southern part of the species' range (Boman et al., 2008). Mortality (immediately following exposure to cold and survival to adulthood), adult weight, and overwintering mortality did not differ between populations. Several mechanisms potentially account for the observed lack of differences, from pre-adaptation to cold from their original area of distribution in a mountainous region in Mexico (Casagrande, 1987) to the presence of trade-offs as observed in *Drosophila simulans* Sturtevant (Watson & Hoffmann, 1996) and the loss of genetic variation when they invaded Europe (Grapputo et al., 2005) with consequent limited ability to respond to selection by cold (Hoffmann & Blows, 1994). We should note that, here, we had only one population from the centre of the European range (Poland) and thus these results are merely indicative. To

study clinal variation more precisely, we would need several populations from different parts of the range as undertaken by, for example, Jenkins & Hoffmann (1999) and Hoffmann et al. (2001, 2002). This limitation should be kept in mind when interpreting our results on between-population differences.

In conclusion, night frost during the growing season per se cannot limit the beetle's northern expansion as it did not result in mortality or small adult size. This suggests higher cold tolerance in *L. decemlineata* than in other insects. *Drosophila serrata* Malloch showed 95% mortality after exposure to −2 °C for 3 h and thus frosts likely limit its distribution (Jenkins & Hoffmann, 1999). High cold tolerance may have enabled *L. decemlineata* to occupy large areas, as predicted by Brown's hypothesis (Brown, 1984) and shown experimentally in *Agabus* beetles (Calosi et al., 2008). We did not take into account the indirect effects of subzero temperatures. Night frost can damage potato leaves (François et al., 1999), reducing beetles' food supply. Reduced food supply would, in turn, decrease beetle performance and, consequently, diminish establishment success. Such effects of subzero temperatures on animals' survival via loss of food have been reported in other species (Inouye, 2000).

The lack of local adaptation does not suggest the inability to increase tolerance through evolutionary processes, but more likely the pre-adaptation to withstand subzero temperatures as discussed above. Low ambient temperature could retard invasion, as it caused high mortality and successful invasion would require a large propagule size (Grevstad, 1999; Memmott et al., 2005). The possibility that periodical lower-than-average summer conditions (13 °C in our experiment) limit the emergence of viable populations cannot be ruled out. Additive genetic variation in life-history traits among families within populations was not estimated in this study. Our earlier studies have shown that *L. decemlineata* have adaptive genetic variation to accelerate their development (Boman et al., 2008; Lyytinen et al., 2008), and thus it is possible that beetles also possess the capacity to improve their ability to grow at low temperatures. At the very least, however, beetles near the northern border of their current range do have a shorter development time than those from the southern part of the range and the beetles have, thus, shown evolutionary capacity to respond to selection by climatic factors (Boman et al., 2008; this paper).

Acknowledgements

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